In vivo analysis of Trk receptor signalling in the mouse nervous system

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Chapter 3

Overlapping and distinct biochemical effects of a juxtamembrane point mutation in *trkB* and *trkC* in the mouse reveals differences in signalling mechanisms between two Trk receptor family members.

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SUMMARY

Neurotrophins regulate development, maintenance and function of vertebrate nervous systems by activation of Trk and p75 receptors. Significant progress has been made in our understanding of the signal transduction events by which Trk receptors mediate their biological functions. In order to compare signalling through two Trk receptors in vivo, we generated mice with a germline mutation in the Shc site in the juxtamembrane region of the TrkC receptor (trkC^{shc} mice), and compared these mice with a similar point mutation in the TrkB receptor (trkB^{shc} mice) (Minichiello et al., 1998). Here, we describe that in primary cortical neurons the phosphorylation of the Akt and Erk1/2 kinases is only partially affected in both receptor mutants. In contrast, in TrkB the Shc-binding site is the major autophosphorylation site, while ligand induced autophosphorylation is hardly affected in TrkC$^{shc/shc}$ neurons. This reduced phosphorylation of the TrkB$^{shc/shc}$ receptor correlates with a shorter lasting interaction of PLCγ with TrkB. We conclude that our point mutant mice highlight a difference in signalling mechanism between TrkB and TrkC.

INTRODUCTION

The neurotrophins are a family of polypeptide growth factors, which use specific receptor tyrosine kinases (the Trk family) to exert their diverse functions in the developing and the mature nervous system (Bibel and Barde, 2000). Specifically, nerve growth factor (NGF) is the preferred ligand for TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) both bind TrkB, and neurotrophin-3 (NT-3) shows high affinity for TrkC, although is also able to signal through TrkA and TrkB (Davies et al., 1995) (Kaplan and Miller, 1997). Studies of mice carrying gene deletion of either neurotrophins or Trk receptors have demonstrated that the neurotrophin/Trk signalling system is required for the survival of different populations of peripheral neurons during development. In the central nervous system neurotrophins support survival and differentiation of selected neuron populations in a partially redundant manner (Minichiello and Klein, 1996) (Alcantara et al., 1997). Finally, in the mature nervous system, neurotrophins can modulate both short-term and long-term synaptic transmission. (Minichiello et al., 1999) (McAllister et al., 1999). It is well established that Trk receptors are structurally similar, and that their ligand-induced dimerization gives rise to autophosphorylation of specific tyrosines in the activation loop of the kinase domain. Subsequent *trans*-phosphorylation of tyrosines in the juxtamembrane and C-terminal regions induces binding of different adaptor proteins that activate well known signalling cascades (Schlessinger, 2000).

Trk receptors contain several conserved tyrosine residues in their intracellular domain, including three in the autoregulatory loop of the kinase domain (Y670, Y674 and Y675 in TrkA) (Bibel and Barde, 2000). The juxtamembrane Y490 of TrkA is located in a NPxY motif and when phosphorylated binds the PTB domain of the Shc adapter molecules (Stephens et al., 1994). Shc is subsequently phosphorylated and recruits the Grb2-Sos complex, which in turn leads to the activation of the Ras-Mapk pathway (Kaplan and Miller, 2000). FRS2 is a multi-domain adapter that competes with Shc for binding to Y490 (Meakin et al., 1999). Furthermore, when phosphorylated after Trk activation FRS2 can recruit Grb2, Crk, Src and the phosphatase SH-PTP-2 (Huang and Reichardt, 2001). By binding Grb2, FRS2 provides another, Shc-independent way of activating the Ras-Mapk cascade. Recruitment of Grb2 also facilitates the activation of PI3-kinase, essential for cell survival in many neurons (Dudek et al., 1997). Despite initial results (Obermeier et al., 1993), the p85 subunit of PI3-kinase does not directly associate with the Trk receptors in primary neurons. At least in some neurons, part of the PI3-kinase activation is achieved by Ras (Rodriguez-Viciana et al., 1997) (Vaillant et al., 1999). However, it is believed that in most neurons the bulk of PI3-kinase activation

is downstream of the multi-adapter binding protein Gab1. After stimulation phosphorylated Grb2 will recruit Gab1, which in turn recruit and activates PI3-kinase (Holgado-Madruga et al., 1997). The PI3-kinase induced activation of Akt is in many neurons critical for survival (Datta et al., 1999). The association of phospholipase C-γ (PLC-γ) with Trk on the C-terminal tyrosine (Y785 in TrkA) regulates intracellular Ca^{2+} levels, although the significance of this pathway for neurotrophin biology remains to be defined (Bibel and Barde, 2000). Taken together these results indicate that the major survival and differentiation promoting pathways are downstream the juxtamembrane Shc adapter binding tyrosine (Huang and Reichardt, 2001). This is clearly the case in TrkA-dependent PC12 cells and primary sympathetic neurons transfected with TrkB (Atwal et al., 2000) (Stephens et al., 1994). Surprisingly, in mice where this tyrosine in TrkB was mutated only a minority of the BDNF-dependent neurons died (Minichiello et al., 1998).

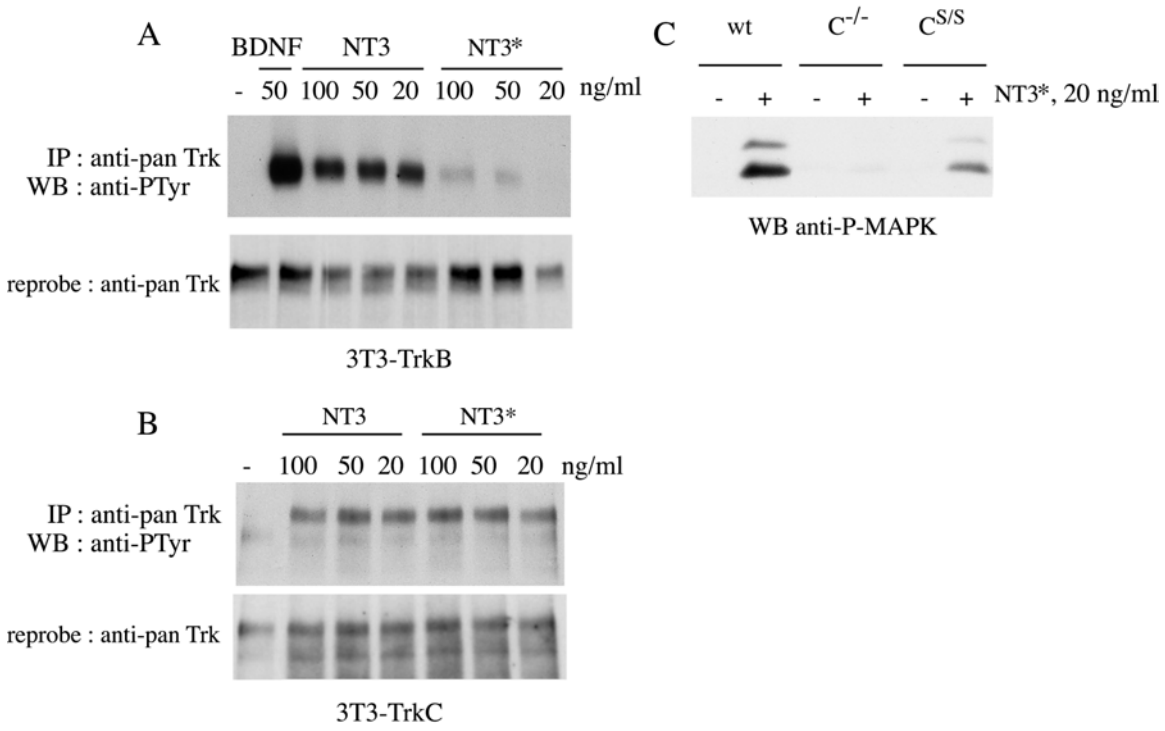

Figure 3-1. Signalling by mutant NT3 in NIH-3T3 fibroblasts and cortical neurons.
(A, B) A mutant version of NT3 (NT3*; see Experimental Procedures) preferably interacts with TrkC (B), and only poorly with TrkB (A). NIH3T3 cell lines stably expressing TrkB or TrkC were stimulated with either wild-type NT3 or NT3* at the indicated concentrations. Cell lysates were immunoprecipitated with anti-pan Trk antibodies and autophosphorylated receptors detected by anti-phosphotyrosine antibodies. At 20 ng/ml, NT3* failed to activate TrkB, whereas wild type NT3 efficiently induced autophosphorylation of TrkB receptors. Blots were reprobed with anti-pan Trk antibodies to control for amounts of immunoprecipitated Trk protein. (C) Primary cortical neuron cultured from wildtype, $trkC^{shc/shc}$ and $trkC^{-/-}$ embryos were stimulated with NT3* at various concentrations. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies against the phosphorylated forms of p42 and p44 ERKs. At 20 ng/ml, NT3* failed to activate MAPK via trkB in $trkC^{-/-}$ neurons (C).

Signaling studies have mostly been performed on TrkA and TrkB in either immortalized PC12 or primary sympathetic neurons in culture (Kaplan and Miller, 2000). In accordance with their sequence similarities, no molecule has thusfar been found to be specifically activated downstream any of the Trk receptors (e.g. (Qian et al., 1998)). Despite significant progress in this area, it remains to be established whether activation of different Trk receptors leads to similar or different biological

outcomes *in vivo*. There are a few examples suggesting that the activation of different Trk receptors or even of the same Trk receptor by two different ligands lead to different biological readouts. Activation of TrkA in sympathetic neurons by NGF or NT3 differentially regulates survival and neuritogenesis (Belliveau et al., 1997). Adenovirally-expressed TrkB uses both PI-3 kinase and Mek to regulate sympathetic neuron survival in vitro, while endogenous TrkA uses PI-3 kinase exclusively (Atwal et al., 2000). BDNF and NT-3 have opposing roles in regulating the growth of basal dendrites of pyramidal neurons in the developing neocortex. This observation suggests interesting differences in signaling capabilities of TrkB and TrkC receptors, although the molecular nature of these differences is unknown (Shieh and Ghosh, 1997) (McAllister et al., 1999). In order to compare signaling through two Trk receptors in vivo, we generated mice with a germline mutation in the Shc site in the juxtamembrane region of the TrkC receptor (trkC^{shc} mice; Chapter 2), and compared these mice with a similar point mutation in the TrkB receptor (trkB^{shc} mice) (Minichiello et al., 1998).

RESULTS

We used standard homologous recombination in embryonic stem (ES) cells to introduce a point mutation into the juxtamembrane exon of the mouse trkC gene (Chapter 2). To investigate the signalling properties of mutant TrkC receptors, we made use of a mutant version of NT3 (here referred to as NT3*), which preferentially interacts with TrkC, but not with the related TrkB or TrkA receptors (Ryden and Ibanez, 1996). To determine receptor specificity, we used NIH3T3 cell lines expressing TrkB or TrkC and stimulated with either wild type NT3 or NT3* prepared from baculovirus-infected insect cells (see Experimental Procedures). While 20-100 ng/ml of wild type NT3 efficiently induced autophosphorylation of TrkC and TrkB receptors, NT3* was a poor activator of TrkB (Figure 3-1A). Stimulation with 20 ng/ml NT3* failed to activate TrkB while its effects on TrkC were similar to wild-type NT3 (Figure 3-1B). To avoid activating endogenous TrkB, we stimulated primary cortical neurons derived from trkC$^{shc/shc}$ mice with 20 ng/ml NT3*. This stimulation was verified to be specific since it did not result in detectable phosphorylation of MAPK in neurons derived from trkC$^{-/-}$ mice (Figure 3-1C). As expected tyrosine phosphorylation of Shc adaptor molecules was not significantly induced after NT3* stimulation (Figure 3-2A). FGF receptor substrate-2 (FRS-2), a known downstream effector of Trk receptors that binds to the juxtamembrane Shc site (Kouhara et al., 1997); (Meakin et al., 1999), was efficiently phosphorylated on tyrosine residues in NT3* or BDNF-stimulated neurons derived from wild type, but greatly reduced in trkC$^{shc/shc}$ or in trkB$^{shc/shc}$ mice (Figure 3-2B). In contrast, binding of the C-terminal-SH2 domain of phospholipase-C (PLCγ) to the phosphorylated C-terminal tyrosine residue in TrkC was unaffected by the Shc site mutation (Figure 3-2C). Also, PLCγ was phosphorylated similarly in wild type and trkC$^{shc/shc}$ neurons stimulated with NT3* (Figure 3-2D).

We next investigated the effects of the Shc site mutation on downstream targets. Trk receptors are known to activate the Ras/MAP kinase pathway, to a large extend by recruiting the Grb2/SOS complex to the Shc site (Bibel and Barde, 2000). We had previously observed that phosphorylation of ERK MAP kinases was reduced and not sustained in BDNF-stimulated neurons derived from trkB$^{shc/shc}$ mutants (Minichiello et al., 1998) (Figure 3-3B). The same result was obtained when cortical neurons from trkC$^{shc/shc}$ mice were stimulated with NT3* (Figure 3-3A). The Shc site also controls the activation of the PI-3K/Akt pathway by recruitment and phosphorylation of the multisite adaptor Gab1 to receptor-bound Shc and FRS2 proteins (Bibel and Barde, 2000). As expected, phosphorylation of Akt was reduced and not sustained in TrkC and TrkB mutant receptors stimulated with NT3* and BDNF-respectively (Figure 3-3A,C). In summary, these results indicate that mutation of the juxtamembrane tyrosine into phenylalanine in the conserved consensus (NPQY) binding site

for signalling proteins that contain a PTB domain in TrkB and TrkC receptors has very similar consequences at least on two downstream signalling events in primary neurons.

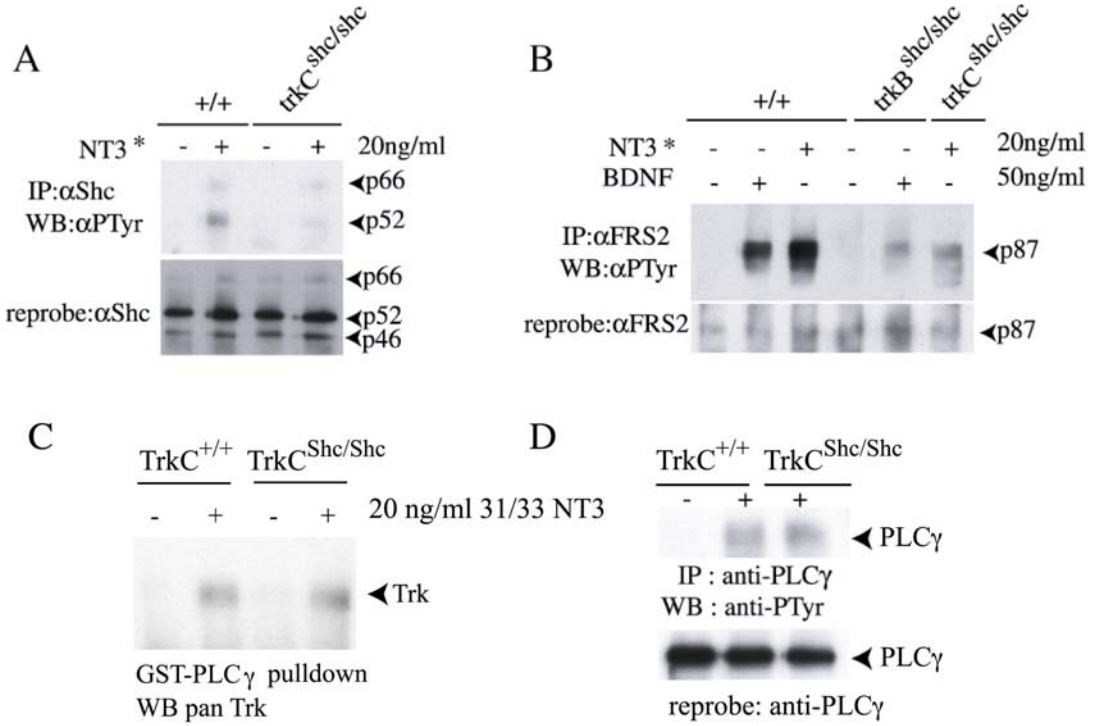

Figure 3-2. Signalling by mutant TrkC^{shc} receptors in cortical neurons.
(A) Tyrosine phosphorylation of Shc proteins. Cortical neurons were stimulated with 20ng/ml of NT3* for 5 minutes. Cell lysates were immunoprecipitated with anti-Shc antibodies followed by immunoblotting with anti-PTyr antibodies. The blot was reprobed with anti-Shc antibodies. (B) Lack of efficient FRS2 binding to mutant TrkC^{shc} receptors. Cortical neurons derived from wild type, trkB$^{shc/shc}$ and trkC$^{shc/shc}$ embryos were stimulated with either 50ng/ml of BDNF or 20ng/ml of NT3* for 5 minutes and lysed. Cell lysates were incubated with anti-FRS2 antibodies followed by immunoblotting with anti-phosphotyrosine antibodies. Weak tyrosine phosphorylation of FRS2 was observed in cortical neurons derived from trkB$^{shc/shc}$ and trkC$^{shc/shc}$ mutants, whereas efficient tyrosine phosphorylation of FRS2 were detected in BDNF or NT3* stimulated wild-type neurons. (C) PLCγ binding to mutant TrkC$^{shc/shc}$ receptors. Wild type and mutant cortical neurons were stimulated with NT3* and lysed. Cell lysates were incubated with the C-terminal SH2 domain of PLCγ coupled to glutathion beads (GST- PLCγ). Bound proteins were immunoblotted with anti-panTrk antibodies. Note efficient binding of activated TrkC^{shc} to PLCγ1 in trkC$^{shc/shc}$ mutant neurons. (D) PLCγ phosphorylation after TrkC$^{shc/shc}$ receptor stimulation. Wild type and mutant cortical neurons were stimulated with NT3* and lysed. Immunoprecipitated PLCγ was immunoblotted with anti-phosphotyrosine (anti-PTyr) antibodies. Blots were reprobed with anti-PLCγ antibodies to control for equal amounts loaded.

We have established that the Shc site in both TrkB and TrkC receptors is only required for survival of a subset of sensory neurons (see Chapters 5, 6, 7). On the other hand, TrkB needs the Shc site to signal proper target innervation by sensory neurons, whereas TrkC is capable of using Shc site-independent mechanisms for target innervation (see Chapters 5 and 6). These findings suggest that TrkB and TrkC receptors are capable of eliciting distinct signalling outputs despite their structural similarities. In order to get more insight into the mechanism that would be responsible for distinct signalling outputs we have tested the requirement of the Shc site for full activation of the two receptors.

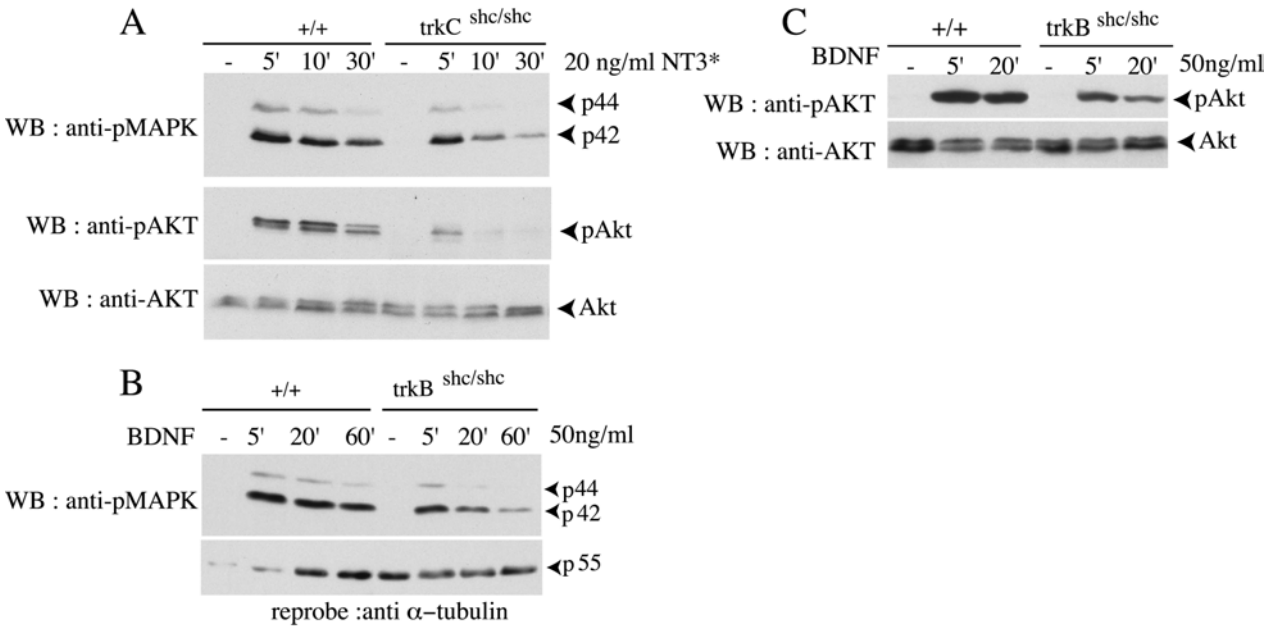

Figure 3-3. Time course of ERK1/2 and AKT phosphorylation in trkC$^{shc/shc}$ and trkB$^{shc/shc}$ mutant cortical neurons. (A) Reduced and short-lived ERK1/2 MAP kinase and AKT phosphorylation in mutant trkC$^{shc/shc}$ cortical neurons. Time course of NT3* stimulation (20 ng/ml) of wild type and mutant neurons. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies against the phosphorylated forms of p42 and p44 ERKs. The blot was reprobed with anti-phospho-AKT antibodies and a second time with antibodies against unphosphorylated AKT to control for the amount of protein loaded in the gel. (B) Reduced and short-lived ERK MAP kinase and (C) AKT phosphorylation in mutant trkB$^{shc/shc}$ cortical neurons. Time course of BDNF stimulation (50 ng/ml) of wild type and mutant neurons. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies against the phosphorylated forms of p42 and p44 ERKs. The blot was reprobed with antibodies against β-tubulin to control for the amount of lysate loaded. Lysates from neurons stimulated with BDNF were also immunoblotted with anti-phospho-AKT antibodies. The blot was reprobed with antibodies against unphosphorylated AKT to control for the amount of lysate loaded.

Surprisingly, mutation of the Shc site impairs TrkB autophosphorylation in response to BDNF (Figure 3-4B and (Minichiello et al., 1998)), but does not impair full activation of TrkC in response to NT3* (Figure 3-4A). This suggests that the unphosphorylated juxtamembrane region of TrkB but not of TrkC has an inhibitory effect on kinase activity. Possibly as a result of partial autoinhibition, we find that PLCγ binding to the other conserved tyrosine in the carboxyterminal region of Trk receptors is reduced in TrkB $^{shc/shc}$. As shown in Figure 3-5A, PLCγ1 is rapidly phosphorylated on tyrosine residues upon stimulation of either wild type TrkC or TrkC$^{shc/shc}$ mutant receptor. Immunoprecipitation of PLCγ1 brings down TrkC$^{shc/shc}$ both at early (1') and later time points (5'). In contrast, association of PLCγ1 and TrkB$^{shc/shc}$ is weak, resulting in loss of co-immunoprecipitation after 5'of BDNF stimulation (Figure 3-5B). This effect is the result of mutation of the Shc site, because wild type TrkB binds PLCγ more robustly and can be co-immunoprecipitated even at very late time points (20'). PLCγ has been shown to be a downstream effector of axonal growth responses (see discussion) raising the possibility that impaired association of PLCγ with TrkB$^{shc/shc}$ receptors contributes to the observed loss of target innervation.

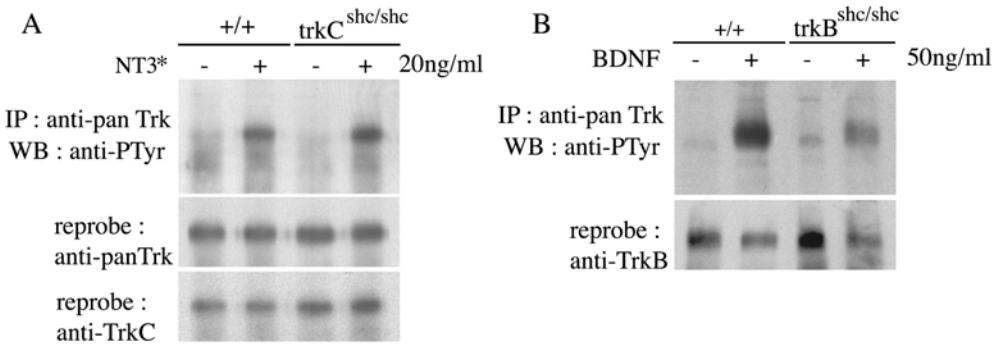

Figure 3-4. Mutation of the Shc site impairs TrkB autophosphorylation in response to BDNF, but does not impair full activation of TrkC in response to NT3*.
(A) Autophosphorylation of TrkC in cortical neurons. Cortical neurons derived from wild type and trkC$^{shc/shc}$ mutants were stimulated with 20ng/ml of NT3* for 5 minutes. Cell lysates were immunoprecipitated with anti-Trk antibodies followed by immunoblotting with anti-phosphotyrosine (anti-PTyr) antibodies. The blot was than reprobed with anti-panTrk antibodies and with anti-TrkC specific antibodies to visualise the levels of TrkC protein. (B) Autophosphorylation of TrkB in cortical neurons. Cortical neurons derived from wild type and trkB$^{shc/shc}$ mutants were stimulated with 50ng/ml of BDNF for 5 minutes. Cell lysates were immunoprecipitated with anti-Trk antibodies followed by immunoblotting with anti-PTyr antibodies. The blot was than reprobed with anti-TrkB to visualise the levels of TrkC protein.

DISCUSSION

In vitro cell systems had previously suggested that two different neurotrophins, BDNF and NT3, presumably acting through TrkB and TrkC receptors, respectively, have very different effects on the same target neuron (Shieh et al., 1997, and references therein). So far, however, biochemical differences in Trk-mediated signalling pathways, which could explain these effects, have not been reported. Furthermore, it is unclear whether similar differences in Trk signalling are present and, more importantly, are required for their biological functions in vivo. To determine whether two Trk receptors use similar or different docking sites for intracellular effectors *in vivo,* we mutated the Shc sites on both TrkB and TrkC receptors using gene targeting technology (Minichiello et al., 1998). Much to our surprise, we found distinct signalling requirements for the Shc site in TrkB and TrkC in sensory neurons (Chapters 5,6,7, this thesis). This may suggest that the observed differences between TrkB and TrkC reflect different signalling properties of the two related receptors. This is not without precedent. Recently, Klinghofer et al. (2001) reported on a study in which the intracellular domains of the highly related α and β isoforms of the platelet-derived growth factor (PDGF) receptor were exchanged using knock-in mice. Mice carrying the αβ hybrid receptors were viable, but suffered from moderate cardiac hypertrophy, suggesting that PDGFβ receptors use additional/distinct intracellular mechanisms compared to the PDGFα receptors (Klinghofer et al., 2001).

Previous results suggest that Trk receptors have highly conserved intracellular domains, and all Trk receptors appear to activate the same set of molecules (Huang and Reichardt, 2001). Despite this, regulation of signals activated by TrkB and TrkC may significantly diverge (Atwal et al., 2000). In order to get more insight into the mechanism that would be responsible for distinct signalling outputs of TrkB versus TrkC we have tested the requirement of the Shc site for full activation of the two receptors. Previous reports have shown that the Shc site in TrkA is the major autophosphorylation site in PC12 cells (Segal et al., 1996.) Surprisingly, mutation of the Shc site impairs TrkB autophosphorylation in response to BDNF, but does not impair full activation of TrkC in response to

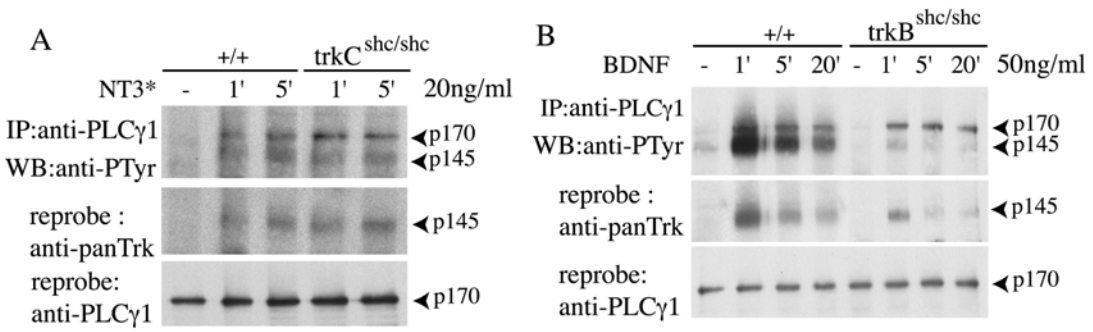

Figure 3-5. Time course of association of PLCγ with Trk receptors in trkB$^{shc/shc}$ and trkC$^{shc/shc}$ cortical lysates.
(A) PLCγ stably binds mutant TrkC^{Shc} receptors. Cortical neurons derived from wild type and trkC$^{shc/shc}$ mutants were treated with NT3* for different length of times. Cell lysates were immunoprecipitated with anti- PLCγ antibodies and immunoblotted with anti-PTyr antibodies. Normal tyrosine phosphorylation was observed for TrkC and PLCγ proteins after 1 minute stimulation as well as at 5 minutes in wild type and trkC$^{shc/shc}$ mutants. The blot was reprobed with anti-panTrk and anti- PLCγ antibodies. (B) Impaired association of PLCγ1 with mutant TrkB^{Shc} receptors. Cortical neurons derived from wild type and trkB$^{shc/shc}$ mutants were treated with BDNF for different length of times. Lysates were immunoprecipitated with anti-PLCγ antibodies and immunoblotted with anti-PTyr antibodies. Normal tyrosine phosphorylation of TrkB and PLCγ was observed in wild type and mutant cells. Co-immunoprecipitation of PLCγ and TrkB was impaired in cells expressing trkB$^{shc/shc}$, compared to cells expressing wild type TrkB. The blot was reprobed with anti-panTrk and anti- PLCγ antibodies.

NT3*. Our data suggest that in the juxtamembrane region of TrkB and TrkC phosphorylation of the tyrosine residue in the consensus sequence NPQY is required for full activation of TrkB, but not for TrkC. There are examples of other receptor tyrosine kinases including the βPDGF receptor whose full activation requires phosphorylation of two tyrosines (579 and 581) in the juxtamembrane region (Baxter et al., 1998). Another example is given by the MuSK receptor (muscle-specific receptor tyrosine kinase) that requires a binding site (NPxY) for PTB domains in adaptor proteins for activation of receptor kinase activity (Zhou et al., 1999). Moreover, Wybenga-Groot et al., (Wybenga-Groot et al., 2001) present structural data showing that the unphosphorylated juxtamembrane region of EphB2 autoinhibits EphB2 kinase activity (see (Hubbard, 2001)). Our data on the mutant Trk receptor suggest that similar autoinhibition may occur in TrkB but not in TrkC. Possibly as a result of partial autoinhibition, PLCγ1 binding to the other conserved tyrosine in the carboxyterminal region of Trk receptors is reduced. PLCγ1 stably binds the mutant TrkC receptor, but dissociates from the mutant TrkB receptors already 5 minutes after BDNF stimulation. PLCγ1 has been shown to be a key downstream effector of axonal growth response mediated by cell adhesion molecules (CAMs) through the FGF receptor (Saffell et al., 1997). It has also been implicated in cytoskeleton reorganisation downstream the PDGF receptor (Yu et al., 1998) . These results correlate with the target innervation phenotype found in the TrkB$^{shc/shc}$ mutants, and thus suggests that impaired stability of PLCγ1 enzyme on TrkB^{shc} receptors can lead to a target innervation defect differently of the TrkC^{shc} receptor. In addition, it is possible that the continued autophosphorylation in the TrkC^{shc} receptors results in the activation of other, unknown substrates of the Trk kinase activity. If these molecules contribute in mediating a threshold signal crucial for target innervation, independent of the adaptors activated via the Shc-site, this could also explain the different phenotypes. To extend our studies and further elucidate the mechanisms that lead to differential regulation of TrkB and TrkC it would be necessary to gain insight into their structural features, as recently described for the EphB2 receptor (Wybenga-Groot et al., 2001).

EXPERIMENTAL PROCEDURES

NIH-3T3 and neuronal cultures. NIH-3T3 fibroblasts stably expressing TrkB or TrkC (Klein et al., 1991) (Lamballe et al., 1993) were grown in DMEM containing 10% calf serum. Cells were serum starved overnight in DMEM containing 0.5% calf serum and stimulated with either BDNF, NT3 (Regeneron Pharmaceuticals, Inc.) or mutant NT3. The mutant NT3 (31/33 NT3) was prepared from baculovirus-infected insect cells as previously described (Ryden and Ibanez, 1996). Neuronal cultures were established from E15.5 mouse cerebral cortices as previously described (Minichiello et al., 1998), derived from intercrosses of wild type, $trkC^{Shc/Shc}$ homozygotes, or $trkB^{Shc/Shc}$ homozygotes.

Biochemistry. NIH-3T3 fibroblasts or cortical neuron cultures were stimulated for different length of time with either 20 ng/ml NT3*, 50 ng/ml of normal NT3 or 50 ng/ml of BDNF. After stimulation the cells were washed twice with ice-cold phosphate-buffered saline, and then lysed on ice for 15 min with either 1% Triton X-100 lysis buffer (50 mM Tris [pH7.5], 120 mM NaCl, 20 mM NaF, 1 mM sodium vanadate, and 1x Protease Inhibitor Cocktail Tablets [Roche]), or in 1% NP-40 lysis buffer (20 mM Tris pH7.4, 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM sodium vanadate, 10 mM NaF, 1 mM NaPP) containing protease inhibitors (10 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM benzamidine and 1 mM PMSF). Insoluble material was removed from the protein extracts by centrifugation at 13,000 rpm for 15' at 4^0C. Immunoprecipitations were performed using specific antibodies for 2h at 4ºC. Blots were probed with specific antibodies and analysed using an ECL chemiluminescence system (Amersham). For the ERK and AKT phosphorylation analysis total cell extracts were separated onto either 15% or 12% SDS-PAGE acrylamide gel respectively, and immunoblotted with specific antibodies.

For the GST pull down experiments cell extracts were incubated with precoupled GST-PLCγ1 fusion protein, consisting of the C-terminal SH2 domain of PLCγ1 (Minichiello et al., 1998). Incubations were left for 3h at 4ºC in the presence of 1 mM sodium vanadate and 2 mM DTT. Immunoprecipitation and immunoblotting was performed using specific anti-antibodies. These included anti-pan Trk polyclonal antibodies (41-4, (Martin-Zanca et al., 1989), C-14, Santa Cruz), anti-TrkB antiserum raised against the kinase domain of TrkB (113-5), anti-phosphotyrosine 4G10 (anti-PTyr) and anti-PLCγ1 monoclonal antibodies (UBI), anti-Shc polyclonal antibody (Transduction Laboratories), anti-FRS2 polyclonal antibody (Santa Cruz), anti-active p44/42 MAP Kinase monoclonal antibody (Biolabs), anti-active pAKT and anti-AKT antibodies (Biolabs), monoclonal anti-β-tubulin (Sigma).

To check TrkC expression in presence or absence of the *neo* gene, whole brains of postnatal day 1 (P1) mice were homogenised with Triton X-100 lysis buffer. After wheat germ agglutinin (WGA) pulldown (Kaplan et al., 1991) or immunoprecipitation with pan-Trk antibodies, blots were probed using specific TrkC antibody 656 (Tsoulfas et al., 1993).